PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 37/00, C12Q 1/68
C12N 5/00, C07H 15/12
A01H 5/00, C12N 15/00

A1

(11) International Publication Number: WO 91/01140

(43) International Publication Date: 7 February 1991 (07.02.91)

(21) International Application Number: PCT/US90/04178

(22) International Filing Date: 25 July 1990 (25.07.90)

(30) Priority data:
385,651 25 July 1989 (25.07.89) US
431,872 6 November 1989 (06.11.89) US

(71) Applicant: CELL GENESYS, INC. [US/US]; 344 Lakeside Drive, Foster City, CA 94404 (US).

(72) Inventors: KUCHERLAPATI, Raju, S. ; 8 Gracie Lane, Darien, CT 06820 (US). KOLLER, Beverly, H. ; 605 Jones Ferry Road, #CC4, Carrboro, NC 27510 (US). SMITHIES, Oliver ; 318 Umstead, Chapel Hill, NC 27514 (US).

(74) Agents: ROWLAND, Bertram, I. et al.; Cooley Godward Castro Huddleson & Tatum, Five Palo Alto Square, 4th Floor, Palo Alto, CA 94306 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: HOMOLOGOUS RECOMBINATION FOR UNIVERSAL DONOR CELLS AND CHIMERIC MAMMALIAN HOSTS

(57) Abstract

Homologous recombination is employed to inactivate genes, particularly genes associated with MHC antigenes. Particularly, the β_2-microglobulin gene is inactivated for reducing or eliminating Class I MHC antigenes. The resulting cells may be used as universal donors. In addition, embryonic stem cells may be modified by homologous recombination for use in producing chimeric or transgenic mammalian hosts.

* See back of page

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MC	Monaco
AU	Australia	FI	Finland	MG	Madagascar
BB	Barbados	FR	France	ML	Mali
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Fasso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GR	Greece	NL	Netherlands
BJ	Benin	HU	Hungary	NO	Norway
BR	Brazil	IT	Italy	PL	Poland
CA	Canada	JP	Japan	RO	Romania
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
DE	Germany	LK	Sri Lanka	TD	Chad
DK	Denmark	LU	Luxembourg	TG	Togo
				US	United States of America

HOMOLOGOUS RECOMBINATION FOR UNIVERSAL DONOR CELLS AND CHIMERIC MAMMALIAN HOSTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of Applications Serial No. 431,872 filed November 6, 1989 and Application Serial No. 385,651, filed July 25, 1989.

INTRODUCTION

Technical Field

The field of the subject invention is the use of homologous recombination to modify and inactivate genes, to produce cells which may serve as universal donors in cellular therapies including transplantation to produce chimeric non-human mammals.

Background

To protect vertebrates from disease and infection, elaborate protective systems have evolved. In mammals, the immune systems serves as the primary defense with many different types of cells and mechanisms to protect the host. A wide variety of hematopoietic cells exist, with the major protective lineages being lymphoid and myeloid. The immune system which results from cells of the lymphoid and myeloid lineages is developed in vivo, so as to recognize self from non-self. Those aberrant situations where the immune system attacks self, such as rheumatoid arthritis, lupus erythematosus, and certain forms of diabetes, are evidence of importance to the host that only foreign agents be attacked. The protective mechanism which protects the host from disease, as a result of invasion of viruses, bacteria,

or other pathogens is also able to recognize cells which come from a different mammalian host, even an allogeneic host.

As part of the system for the self versus foreign recognition, the surface membrane protein major histocompatibility complex (MHC) antigens serve an important role. Each host has a personal set of Class I and II MHC antigens, which serve to distinguish that host from other hosts. The lymphoid system is predicated upon recognition of the presence of such MHC antigens as self. Where transplantation from another allogeneic host occurs, unless the transplant is matched with the host or the host is immunocompromised, the transplant may be attacked and destroyed by the immune system. When a transplant occurs which includes lymphocytes, monocytes or progenitors thereof, particularly bone marrow, a graft may attack the host as foreign, resulting in graft-versus-host disease.

There are many situations where one may wish to transplant cells into a recipient host when the recipient's cells are missing, damaged or dysfunctional. When the host is immunocompromised, there may be an interest in transfusing specific white cells, particularly T-cells, which may protect the host from various diseases. When the host lacks the ability to raise a defense against a particular disease, there may also be an interest in administering specific T-cells or B-cells or precursors thereof which may supplement the host's compromised immune system. In other cases, where certain cells are lacking, such as islets of Langerhans in the case of diabetes, or cells which secrete dopamine in the case of Parkinson's disease, or bone marrow cells in various hematopoietic diseases, or muscle cells in muscl wasting diseases or retinal epithelial cells in visual disorders, it would be desirable to be able to provid cells which

could fulfill the desired function. In order for the cells to be effective, they must be safe from attack by the host, so that they may function without being destroyed by the immune system. It is therefore of interest to find effective ways to produce cells which may function, proliferate, and differentiate as appropriate, while being safe from attack by a recipient's immune system.

There is also substantial interest in being able to study various physiological processes in vivo in animal models. In many of these situations, one would wish to have a specific gene(s) inactivated or introduced in a site-directed fashion. Where all or a substantial proportion of the cells present in the host would be mutated, the various processes could be studied. In addition, heterozygous hosts having one wild-type gene and one mutated gene could be mated to obtain homozygous hosts, so that all of the cells would have the appropriate modification. Such genetically modified animals could serve for screening drugs, investigating physiologic processes, developing new products, and the like.

Relevant Literature

A number of papers describe the use of homologous recombination in mammalian cells, including human cells. Illustrative of these papers are Kucherlapati et al., Proc. Natl. Acad. Sci. USA 81:3153-3157, 1984; Kucherlapati et. al., Mol. Cell. Bio. 5:714-720, 1985; Smithies et al., Nature 317:230 234, 1985; Wake et al., Mol. Cell. Bio. 8:2080-2089, 1985; Ayares et. al., Genetics 111:375-388, 1985; Ayares et. al., Mol. Cell. Bio. 7:1656-1662, 1986; Song et. al., Proc. Natl. Acad. Sci. USA 84:6820-6824, 1987; Thomas et. al., Cell 44:419-428, 1986; Thomas and Capecchi, Cell 51: 503-512, 1987; Nandi et. al., Proc. Natl. Acad. Sci. USA 85:3845-3849, 1988; and Mansour et. al., Nature 336:348-352, 1988.

Evans and Kaufman, Nature 294:154-146, 1981; Doetschman et al., Nature 330:576-578, 1987; Thomas and Capecchi, Cell 51:503-512, 1987; Thompson et al., Cell 56:316-321, 1989; individually describe various aspects of using homologous recombination to create specific genetic mutations in embryonic stem cells and to transfer these mutations to the germline. The polymerase chain reaction used for screening homologous recombination events is described in Kim and Smithies, Nucleic Acids Res. 16:8887-8903, 1988; and Joyner et al., Nature 338:153-156, 1989. The combination of a mutant polyoma enhancer and a thymidine kinase promoter to drive the neomycin gene has been shown to be active in both embryonic stem cells and EC cells by Thomas and Capecchi, supra, 1987; Nicholas and Berg (1983) in Teratocarcinoma Stem Cell, eds. Siver, Martin and Strikland (Cold Spring Harbor Lab., Cold Spring Harbor, NY (pp. 469-497); and Linney and Donerly, Cell 35:693-699, 1983.

SUMMARY OF THE INVENTION

Mutated mammalian cells are produced by homologous recombination, where the cells may be used as universal donor cells as a result of loss of MHC antigens or the cells are embryonic stem cells which may be used to produce chimeric mammals carrying this mutation. Particularly, by inactivating at least one allele of at least one MHC antigen chain, e.g., β_2-microglobulin, cells can be produced which have reduced capability for expression of MHC antigens and can be further used for complete removal of expression of at least one type of MHC antigen. The resulting cells having reduced expression of MHC antigens may be used as universal donors lacking markers for host (recipient) immune attack. The cells may also be used in vitro to interact with other cells. The chimeric animals carrying this trait may be used in the study of immunodeficiency.

DESCRIPTION OF SPECIFIC EMBODIMENTS

Homologous recombination is employed for inactivation or alteration of genes in a site-directed manner, particularly a gene associated with an MHC antigen. Depending upon the nature of the cell, the cell lacking at least one competent MHC antigen may find use as a donor to an allogeneic host or if an embryonic stem cell, may find use in the production of chimeric mammalian hosts.

Of particular interest is the inactivation of at least one, preferably both, copies of a subunit of an MHC antigen, more particularly, β_2-microglobulin. Where a mutation in the β_2-microglobulin gene of an embryonic stem cell is produced, a mammalian host derived from the embryonic stem cell may be used for investigation of the immune system and the role of Class I MHC antigen in that system. Of particular interest are methods which provide for cells lacking at least one MHC antigen, Class I or Class II, preferably Class I, which cells may serve a variety of functions in a viable host. The method involves transfection of mammalian cells, particularly normal cells, of a predetermined species with DNA associated with one of the loci related to the β_2-microglobulin gene, the α-subunit(s) of the Class I or Class II MHC antigens or the β-subunit(s) of the Class II MHC antigens. The human Class II MHC antigens are HLA-DR, DP AND DQ, where DR is of primary interest.

The DNA will comprise at least a portion of the gene(s) at the particular locus with introduction of a lesion into at least one, usually both copies, of the native gene(s), so as to prevent expression of a functional MHC antigen molecule. The lesion may be an insertion, deletion, replacement or combination thereof. When the lesion is introduced into only one copy of the gene being inactivated, the cells having a

single unmutated copy of the target gene are amplified and may be subjected to a second transformation, where the lesion may be the same or different from the first lesion, usually different, and where a deletion, or replacement is involved may be overlapping at least a portion of the lesion or allele. The resulting transformants are screened for the absence of a functional target antigen and the DNA of the cell may be further screened to ensure the absence of a wild-type target gene. Alternatively, homozygosity as to a phenotype may be achieved by breeding hosts heterozygous for the mutation.

The cells which may be subjected to transformation may be any mammalian cells of interest, which may find use in cell therapy, research, interaction with other cells in vitro or the like. Cells of particular interest include among other lineages the islets of Langerhans, adrenal medulla cells which may secrete dopamine, osteoblasts, osteoclasts, epithelial cells, endothelial cells, T-lymphocytes, neurons, glial cells, ganglion cells, retinal cells, embryonic stem cells, liver cells, bone marrow cells, and myoblast (muscle) cells.

These cells will be selected to achieve a particular function and be introduced into a mammalian host or used for research or other purpose. Also of interest will be the stem cells which act as the progenitors for any of the above cells, which may be the original progenitor or a progenitor cell which is already dedicated to a particular lineage. Of particular interest will be epidermal cells, such as keratinocytes, and retinal epithelial cells, and myoblasts and hematopoietic cells and other cells which may be readily manipulated in vitro, maintained for long periods of time in culture and may be introduced into a host, wher the cells will remain viable and functional for long periods of time.

For embryonic stem cells, an embryonic stem cell line may be employed or embryonic stem cells may b obtained freshly from a host. The cells may be grown on an appropriate fibroblast-feeder layer or grown in the presence of leukemia inhibitory factor (LIF) and then used for mutation.

The procedures employed for inactivating one or both copies of a particular MHC antigen will be similar, differing primarily in the choice of sequence, selectable markers used, and the method used to identify the absence of the MHC antigen, although similar methods may be used to ensure the absence of expression of a particular antigen. Since the procedures are analogous, the inactivation of the β_2-microglobulin gene will be used as an example. It is to be understood that substantially the same procedures but with other genetic sequences will suffice for the α- and β-subunits of the Class II MHC antigens.

DNA constructs may be employed which provide for the desired introduction of the lesion into the cell. The constructs may be modified to include functional entities other than the mutated sequence which may find use in the preparation of the construct, amplification, transformation of the host cell, and integration of the construct into the host cell. Techniques which may be used include calcium phosphate/DNA coprecipitates, microinjection of DNA into the nucleus, electroporation, bacterial protoplast fusion with intact cells, transfection, or the like. The DNA may be single or double stranded, linear or circular, relaxed, or supercoiled DNA. For various techniques for transforming mammalian cells, (see Keown et al., Methods in Enzymology).

The homologous sequence for targeting the construct may have one or more deletions, insertions, substitutions or combinations th reof. For example, the β_2-microglobulin may include a deletion at one

site and an insertion at another site which includes a gene which may be used for selection, where the presence of the inserted gene will result in a defective inactive protein product. Preferably, replacements are employed. For an inserted gene, of particular interest is a gene which provides a marker, e.g., antibiotic resistance such as neomycin resistance, including G418 resistance.

The deletion will be at least about 50 bp, more usually at least about 100 bp, and generally not more than about 20 kbp, where the deletion will normally include at least a portion of the coding region including a portion of or one or more exons, a portion of or one or more introns, and may or may not include a portion of the flanking non-coding regions, particularly the 5'-non-coding region (transcriptional regulatory region). Thus, the homologous region may extend beyond the coding region into the 5'-non-coding region or alternatively into the 3'-non-coding region. Insertions will generally not exceed 10 kbp, usually not exceed 5 kbp, generally being at least 50 bp, more usually at least 200 bp.

The homologous sequence should include at least about 100 bp, preferably at least about 150 bp, more preferably at least about 300 bp of the target sequence and generally not exceeding 20 kbp, usually not exceeding 10 kbp, preferably less than about a total of 5 kbp, usually having at least about 50 bp on opposite sides of the insertion and/or the deletion in order to provide for double crossover recombination.

Upstream and/or downstream from the target gene construct may be a gene which provides for identification of whether a double crossover has occurred. For this purpose, the herpes simplex virus thymidine kinase gene may be employed, since the presence of the thymidine kinase gene may be detected by the use of nucleoside analogs, such as acyclovir or

gancyclovir, for their cytotoxic effects on cells that contain a functional HSV-tk gene. The absence of sensitivity to these nucleoside analogs indicates the absence of the thymidine kinase gene and, therefore, where homologous recombination has occurred that a double crossover event has also occurred.

The presence of the marker gene inserted into the β_2-microglobulin gene establishes the integration of the target construct into the host genome. However, DNA analysis will be required in order to establish whether homologous or non-homologous recombination occurred. This can be determined by employing probes for the insert and then sequencing the 5' and 3' regions flanking the insert for the presence of β_2-microglobulin extending beyond the flanking regions of the construct or identifying the presence of a deletion, when such deletion is introduced.

The polymerase chain reaction may be used with advantage in detecting the presence of homologous recombination. Primers may be used which are complementary to a sequence within the construct and complementary to a sequence outside the construct and at the target locus. In this way, one can only obtain DNA duplexes having both of the primers present in the complementary chains if homologous recombination has occurred. By demonstrating the presence of the probe sequences or the expected size sequence, the occurrence of homologous recombination is supported.

The construct may further include a replication system which is functional in the mammalian host cell. For the most part, these replication systems will involve viral replication systems, such as Simian Virus 40, Epstein-Barr virus, papilloma virus, adenovirus and the like.

Wher a marker gene is involv d, as an insert, and/or flanking gene, depending upon the

nature of the gene, it may have the wild-type transcriptional regulatory regions, particularly the transcriptional initiation regulatory region or a different transcriptional initiation region. Whenever a gene is from a host where the transcriptional initiation region is not recognized by the transcriptional machinery of the mammalian host cell, a different transcriptional initiation region will be required. This region may be constitutive or inducible, preferably inducible. A wide variety of transcriptional initiation regions have been isolated and used with different genes. Of particular interest as promoters are the promoters of metallothionein-I and II from a mammalian host, thymidine kinase, β-actin, immunoglobulin promoter, human cytomegalovirus promoters, and SV40 promoters. In addition to the promoter, the wildtype enhancer may be present or an enhancer from a different gene may be joined to the promoter region.

The construct may further include a replication system for prokaryotes, particularly E. coli, for use in preparing the construct, cloning after each manipulation, allowing for analysis, such as restriction mapping or sequencing, followed by expansion of a clone and isolation of the plasmid for further manipulation. When necessary, a different marker may be employed for detecting bacterial transformants.

Once the vector has been prepared, it may be further manipulated by deletion of the bacterial sequences as well as linearization, where a short deletion may be provided in the homologous sequence, generally not exceeding about 500 bp, generally being from about 50 to 300 bp. The small deletion will generally be near one or other end of the targeted structural gene.

Once the construct has b en prepared and manipulated and the undesired sequences removed from

the vector, e.g., the undesired bacterial sequences, the DNA construct is now ready to be introduced into the target cells. As already indicated, any convenient technique for introducing the DNA into the target cells may be employed. After transformation of the target cells, many target cells are selected by means of positive and/or negative markers, as previously indicated, neomycin resistance and acyclovir or gancyclovir resistance. Those cells which show the desired phenotype may then be further analyzed by restriction analysis, electrophoresis, Southern analysis, polymerase chain reaction or the like. By identifying fragments which show the presence of the lesion(s) at the target gene site, one can identify cells in which homologous recombination has occurred to inactivate one of the two copies of the target gene.

The second construct will differ from the first construct in not necessarily requiring a marker for selection, since the absence of the target MHC antigen on the surface of the cells may be used as a marker. Thus, one may again use insertions, deletions or replacements as lesions for modifying and inactivating the target gene. Similarly, one may detect the absence of a Class II MHC antigen on the surface as evidence of the absence of expression of the particular Class II MHC antigen.

Transformation of the cells in which one of the copies has been inactivated may then be performed in the same or different way from the previous method of transformation. The resulting transformed cells may then be selected by the absence of the target MHC antigen on the surface of the cell. This can be achieved in a variety of ways. For example, one may use antibodies to any epitope of the target MHC antigen in conjunction with complement to kill any cells having the antigen. Alternatively, one may use conjugates of the appropriate antibody, particularly

monoclonal antibody with a toxin, such as the A chain of ricin, abrin, diphtheria toxin, or the like. Affinity chromatography may be employed, where antibodies may be used to remove cells comprising the target antigen. The resulting cells which survive should be free of at least one MHC antigen on their surface and now not be as subject to transplant rejection when introduced in vivo as wild-type cells.

The resulting cells will then be screened to ensure that substantially no Class I MHC antigens are on the surface. This may be achieved as described above by selecting for cells lacking the Class I MHC antigen. The cells may then be grown in an appropriate nutrient medium for expansion and used in a variety of ways. For example, with keratinocytes, the cells may be used for replacement of skin in the case of burns, where keratinocytes may be grown to form a continuous layer prior to application. Similarly, the keratinocytes may be used in the case of plastic surgery to replace skin removed from the host for use at another site. Other uses for the keratinocytes include transplantation in decubitus ulcers.

In the case of islets of Langerhans, they may be grown and introduced into capsules or otherwise for insertion into a host for the production of insulin. In the case of retinal epithelial cells, they could be injected into the subretinal space of the eye to treat visual disorders such as macular degeneration. In the case of immune cells, they could be injected into the bloodstream or elsewhere to treat immune deficiency. In the case of myoblasts they could be injected at various sites to treat muscle wasting, such as Duchenne's muscular dystrophy.

Depending upon the nature of the cells, the therapy involved, and the disorder, the c lls may be employed as films, introduced in containers for maintenance at a particular site, or as solid masses

impregnated in inert matrices or independent of a matrix. The number of cells administered will vary widely, depending upon the particular application and the manner in which the cells are administered.

Administration may be by injection, topical application, incision and placement, in the appropriate location.

For embryonic stem cells, after mutation, the cells may be plated onto a feeder layer in an appropriate medium, e.g., fetal bovine serum enhanced DMEM. Cells containing the construct may be detected by employing a selective medium and after sufficient time for colonies to grow, colonies may be picked and analyzed for the occurrence of homologous recombination. As described previously, the polymerase chain reaction may be used, with primers within and without the construct sequence but at the target locus. Those colonies which show homologous recombination may then be used for embryo manipulating and blastocyst injection. Blastocysts may be obtained from 4 to 6 week old superovulated females by flushing the uterus 3.5 days after ovulation. The embryonic stem cells may then be trypsinized and the modified cells added to a droplet containing the blastocysts. At least one, usually at least about 10, and up to about 30 of the modified embryonic stem cells may be injected into the blastocoel of the blastocyst. After injection, at least one and not more than about 15 of the blastocysts are returned to each uterine horn of pseudopregnant females. Females are then allowed to go to term and the resulting litters screened for mutant cells having the construct. The blastocysts are selected for different parentage from the transformed ES cells. By providing for a different phenotype of the blastocyst and the ES cells, chimeric progeny can be readily detected. A particularly useful phenotype is hair color, although any phenotype may be used or, if desired, one may look

to genotype, probing for the presence of the modified genomic DNA.

The pups will usually be born 16-18 days after introduction of the blastocysts into foster mothers. The chimeric animals are screened for the presence of the transformed genome and males and females comprising the transformed genome are mated. The homozygous progeny lack Class I MHC cells and mature CD8 T-cells (TCR $\alpha\beta$).

The mammals may be any non-human mammal, such as laboratory animals, domestic animals, pets, etc.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

PROLIFERATION OF EPIDERMAL KERATINOCYTES LACKING MHC ANTIGEN DUE TO INACTIVATION OF β_2-MICROGLOBULIN GENE EXPRESSION

Cells

Mouse epidermal keratinocytes are obtained from the skin of a newborn mouse. The skin samples are rinsed in serum-free medium and minced into small fragments. The fragments are treated with trypsin and the resulting single cell suspension washed and plated on 3T3 fibroblast feeder layers. EGF (5 ng/ml) is added at the end of five days. The cells are maintained in media supplemented with hydrocortisone (10^{-6}M), cholera toxin (10^{-7}M), insulin (5 ng/ml), transferrin (5 ng/ml) T3 (2×10^{-8}M) and 20% fetal calf serum. Unused cells are stored in liquid nitrogen.

Human epidermal keratinocytes are isolated using a fresh skin sample from a circumcised skin as the source of the keratinocytes. The sample is then treated substantially as described above.

DNA Vectors

The mouse and human β_2-microglobulin genes as isolated and characterized by Parnes and Seidman, Cell 29:661-669, (1982) and Gussow, et al., J. Immunol, 139:3132-3138 (1987), respectively, are employed for homology.

Construction of Inactivation Vector 1

The inactivation vectors are constructed from 4kb HindIII fragment of the genomic DNA which encompasses the second, third and fourth exons of the β_2-microglobulin gene. The 4kb HindIII subcloned into pBR322 is digested with EcoRI and the selectable neomycin phosphotransferase (neo^R) gene inserted. The neo^R gene is obtained from pSV2neo (Southern and Berg, Mol. Appl. Genet. 1:332, (1982)). The resulting vector is called B2KO1.

Construction of Inactivation Vector 2

The starting plasmid for the construction of the second vector is B2KO1. In this case, the herpes simplex virus type 1 thymidine kinase gene is inserted at the HindIII site of B2KO1.

Inactivation of One Copy of β_2-Microglobulin

The DNA which is used for transformation in the first or second stage comprises the inserted sequence with flanking homologous sequences from the cloning plasmid B2KO1 and the same sequence flanked at one end by tk gene free of the bacterial plasmid DNA. The resulting DNA fragments are purified by ethanol precipitation and cleared by passage through a 0.22 micron filter. The DNA is isolated by conventional means and introduced into the keratinocyte cells by microinjection (Capecchi, Cell 22:479-488 (1980). Approximat ly 5-50 copies of the DNA constructs are injected into each nucleus. The cells are then grown ln selective medium comprising 200 μg/ml of G418

(Geneticin, Gibco Labs). For the second construct, the cells are also plated in Gancyclovir (Syntex Corp, Palo Alto, CA) or Ayclovir (Burrows-Wellcome, Research Triangle Park, NC). Cells from colonies are isolated and analyzed by the polymerase chain reaction and Southern blot hybridization. Cells demonstrating one copy of the β_2-microglobulin being inactivated are used for knocking out the second copy.

Inactivation of The Second Copy of the β_2-Microglobulin Gene

Gene

Cells obtained from above with a single inactivated β_2-microglobulin gene are microinjected as described above with the modified B2K02 plasmid and cells resistant to Gancylovir or Acyclovir isolated. Cells which lack Class I gene expression are isolated by combining the cells with monoclonal antibodies specific for β_2-microglobulin and complement as described by Parish et al. (1974) Eur. J. Immunol. 4:808. Resulting viable cells are grown in selected medium and passed through an affinity column of the same monoclonal antibodies. The column is prepared as described by Harlow and Lane, 1988, Antibodies: A Laboratory Manual, CSH Press. Southern blot analysis of the cells is performed to establish the proper locus of integration. The cells are then expanded and stored for further use.

Generation of Monolayer of Keratinocytes

The resulting cells lacking Class I MHC are used to grow a monolayer of keratinocytes as described by Rheinwald and Green, Cell 6:331-343, 1975. This layer is transplanted onto allogenic mice as described by Rheinwald and Green, 1975, supra. The cells adhere to the surface and grow to provide a prot ctive skin layer.

Following the same procedure as described above for β_2-microglobulin the HLA-DR genes may be inactivated by employing homologous sequences flanking the a-subunit of the HLA-DR gene of the host cell. In this way cells which have the Class II MHC antigen or may have the capability to have the expression of such antigen induced are prevented from expressing the primary Class II antigen associated with the cellular immune response.

In the next study, embryonic stem cells were modified by homologous recombination with one of the β_2-microglobulin genes.

Materials and Methods

Construction of the Targeting Plasmid

The plasmid pKCβ_2B contains the entire β_2m gene within an 8.4 kbp XhoI fragment (Ozato and Orrison, Proc. Natl. Acad. Sci USA 82:2427-2431, 1985; Warner et al., Bio. Reprod. 36:611-616, 1987). The 5'XhoI to BamHI fragment of this gene was subcloned into pUC19. Two KpnI restriction enzyme sites, one in the 5' flanking DNA and the other within the first intron, were removed by digestion with KpnI followed by treatment with T4 polymerase and re-ligation. A unique ClaI site was created in exon 2 by partial digestion with EcoRI followed by treatment with Klenow polymerase and ligation with ClaI linkers. The 1150 bp XhoI to HI fragment of the plasmid pMC1 Neo (Kim and Smithies, Nucleic Acid Res. 16:8887-8903, 1988), containing a neomycin gene driven by the Herpes simplex virus thymidine kinase gene (HSV-tk) promoter and a polyoma enhancer, was inserted via linkers into this ClaI site. Two plasmids, C65.2.3 and C65.5.9, were obtained that differed in the transcriptional orientation of th inserted fragment with respect to that of the β_2-microglobulin gene. The 5' XhoI to KpnI fragment of each of these was

cloned into pUC19 in order to obtain the targeting vectors used in our experiments. In plasmid C84.4B the 5′ to 3′ orientation of the neomycin and β_2m promoters is identical. The opposite configuration occurs in plasmid C84.2D.

Culturing, Electroporation, and Selection of ES Cells

The ES cell line E14TG2a (Sawicki et al., Nature 294:450-451, 1981) was cultured on mitomycin-treated primary embryonic fibroblast-feeder layers essentially as described (Ostrand-Rosenberg et al., Proc. Natl. Acad. Sci. 86:5084-5088, 1989). The embryonic fibroblasts were prepared from embryos from C57BL/6 females that had mated 14 to 17 days earlier with a male homozygous for a neomycin transgene (Evans and Kaufman, Nature 292:154-156, 1981); these cells are capable of growth in media containing G418. Electroporation conditions were similar to those that have been described previously (Doetschman et al., Nature 330:576-578, 1987). ES cells were trypsinized, resuspended in culture media at a concentration of $4x10^7$/ml and electroporated in the presence of the targeting DNA at a concentration of 12nM in the first experiment and 5nM DNA in the second. A voltage of 300 V with a capacitance of 150-250 μF was found optimal with an electroporation cell of 5 mm length and 100 mm^2 cross section. $5x10^6$ electroporated cells were plated onto mitomycin-treated fibroblasts in 100 mm dishes in the presence of Dulbecco's modified Eagle's media (DMEM) supplemented with 15% fetal bovine serum (FBS) and 0.1 mM 2-mercaptoethanol. The media was replaced 24 hr after electroporation with media containing 200 μg/ml G418.

Analysis of G418 Resistant ES Cell Colonies

ES colonies visible 10-14 days after electroporation were picked with drawn out capillary pipettes for analysis using the polymerase chain

reaction (PCR). Half of each picked colony was saved in 24-well plates already seeded with mitomycin-treated feeder cells. The other halves, combined in pools of 3-4, were transferred to Eppendorf tubes containing approximately 0.5 ml of PBS and analyzed for homologous recombination by PCR. Conditions for PCR reactions were essentially as described (Linney and Donerly, Cell 35:693-699, 1983). The ES cells were pelleted, resuspended in 5 μl of phosphate buffered saline (PBS), and lysed by the addition of 55 μl of H_2O to each tube. DNAses were inactivated by heating each tube at 95°C for 10 min. After treatment with proteinase K at 55°C for 30 min, 30 μl of each lysate was transferred to a tube containing 20 μl of a reaction mixture including PCR buffer, 1.5 μg of each primer, 3U of Taq polymerase, 10% DMSO, and dATP, dCTP, dGTP and dTTP each at 0.2 mM. PCR was carried out for 55 cycles using a thermocycler modelled after one described previously (Kim and Smithies, supra, 1988), with 65 seconds melt at 92°C and a 10 min annealing and extension time at 65°C. The two priming oligonucleotides, TGGCGGACCGCTATAGGAC and GATGCTGATCACATGTCTCG, correspond respectively to sequences located 650 bases 3' of the start codon of the neomycin gene and sequences located in exon 3 of the β_2m gene. 20 μl of the reaction mix was electrophoresed on agarose gels and transferred to nylon membranes (Zeta Bind). Filters were probed with ^{32}P-labelled 450 bp EcoRI to KpnI fragment of the β_2m gene.

Preparation and Restriction Enzyme Analysis of Genomic DNA

Genomic DNA was prepared from ES cells, whole new born mice, and mouse tails by conventional methods. DNA was digested with restriction enzymes as directed by the manufacturers and fragments were separated on 0.7% agarose gels. DNA was transferred

to nylon membranes and probed with the ^{32}P labelled fragment described above.

Embryo Manipulation and Blastocyst Injection

Mice were purchased from either Jackson Laboratories (Bar Harbor, ME) or Charles River (Raleigh, NC). C57BL/6 blastocysts were obtained from 3 to 4 week old superovulated females. Uteri were flushed with M2 media (Joyner et al., Nature 338, 153-156, 1989) 3.5 days after ovulation. Blastocysts were collected, washed several times in fresh M2 media, and placed in a 100 μl droplet of M2 under paraffin oil. ES cells were trypsinized, washed once with fresh DMEM media and diluted to approximately $2x10^6$ cell/ml. 5 μl of cells were added to the droplet containing the blastocysts. 10 to 15 ES cells were injected into the blastocoel of each blastocyst. Following injection 6 to 9 blastocyst were returned to each uterine horn of pseudopregnant females mated 2.5 days previously with vasectomized males. Both C57BL/6 x DBA F_1 and C57BL/6 x CBA F_1 mice proved to be excellent foster mothers, yielding a pregnancy rate close to 100% and able to raise small litters.

Isolation and Characterization of Targeted ES cells

Two independent targeting experiments were carried out. In each, $2x10^7$ cells were electroporated in the presence of the incoming DNA, and were then cultured in media containing G418. After about two weeks, G418 resistant colonies were readily apparent. A portion of each colony was then transferred to an individual well of a 24-well plate, while the remaining portion was pooled with portions from two to four other colonies for PCR analysis. In the first experiment, one pool gave a positive PCR signal out of 32 pools that included a total of 100 G418 resistant colonies. The three individual colonies that had

contributed to this positive pool were analyzed individually by PCR, and a positive clone, ES39B, was identified. Similar analysis of 134 G418 resistant colonies obtained in the second experiment also yielded a clone, ES22A, which generated the 910 bp DNA fragment indicating successful targeting when subjected to PCR.

In order to verify the targeted disruption of one copy of the β_2m gene, (the gene is autosomal and present in two copies), the two PCR positive clones, ES39B and ES22A, were expanded, and their DNA was isolated and then analyzed by Southern blotting using a probe that detects sequences from the second exon and part of the first intron of the β_2m gene. Patterns obtained with the restriction enzymes XbaI, BamHI and KpnI match those expected if one of the two copies of the β_2m gene had been disrupted in the planned manner in the PCR-positive clones. That is, one DNA fragment identical in size to that present in untreated cells, was present in untreated cells, but of decreased intensity in the PCR positive clones, with all three enzymes. An additional fragment of the size predicted for a homologous recombination event was present only in the PCR-positive clones. The insertion of the neomycin gene in the second exon by the recombination results in an XbaI fragment detectable with the β_2m specific probe that is approximately 1 kb longer than the equivalent fragment in the native locus. A new BamHI site is introduced into the locus by the targetting DNA, reducing the size of the BamHI fragment detected by the β_2m probe from 10.6 kbp to 900 bp. A new fragment is also seen after KpnI digestion. In ES39B the KpnI fragment is 7 kb in length, as predicted by a crossover between the 5′ end of the targeting plasmid and the native locus. In ES22A this new KpnI fragment is 4.0 kb in length, which indicates that the deleted KpnI sites were not incorporated into the locus. This

observation indicates that one of the crossovers in cell line ES22A resolved between the third KpnI site of the native locus and the inserted neomycin gene of the incoming DNA, presumably after branch migration of a crossover intermediate. Although the 5' crossover sites differ, both modified cell lines now contain a β_2m gene disrupted in the planned way by insertion of a neomycin gene in exon 2. Re-hybridization of the filter used for the autoradiography with a probe for the neomycin gene shows that the only bands that hybridize are those predicted by the structure of the construct.

Chimeric Offspring of Targeted ES Cells

The two ES cell lines carrying the inactivated β_2m genes are expected to allow the introduction of this mutation into the mouse germline. Toward this end, we injected 10 to 15 cells into C57BL/6 blastocysts. Embryos were reimplanted into pseudopregnant females. Because the ES cell line E14TG2a was isolated from strain 129/01a embryos, it and all cell lines derived from it are expected to carry the coat color markers characteristic of this mouse strain. These include the dominant A^w allele at the agouti locus, the recessive chinchilla allele at the c-locus, and the recessive p-allele (pink-eyed dilution) at the p-locus (Quinn et al., J. Reprod. Fertil. 66:161-168, 1981). Contribution of ES cells to the mesoderm-derived portions of hair follicles results in an agouti coat. Hair follicles to which melanocytes of ES cell origin (and therefore carrying the p and c^{ch} mutations) have migrated produce cream colored hairs. Both of these coat colors are easily distinguished from the solid black coat seen in pups derived from non-agouti C57BL/6 host blastocysts.

More than 70% of surviving pups are chimeras. The intensity of the 6.1 XbaI band diagnostic of the targeted β_2m locus shows that the

modified ES cells contributed extensively to the tissue of this animal.

Generation of Chimeric Mice

Three to four week old C57BL/6 female mice were superovulated by the sequential injection of PMS and hCG and mated with fertile males of similar strain. Four days after mating, the female mice were sacrificed, and blastocysts obtained by flushing the uterus with M9 media. The collected blastocysts were transferred to a droplet of the same media that was submerged in paraffin oil and also contained some ES22a cells. These cells had been prepared for injection by trypsinization followed by washing and resuspending in M2 media. Ten to fifteen ES22a cells were introduced into the blastocoel of each blastocyst using standard micromanipulation techniques. The ES cell containing blastocysts were then transferred to the uterus of a pseudopregnant foster mother. Foster mothers were obtained by mating B6/D2 females with vasectomized male mice. Females which had mated 2.5 days prior to the date of transfer, as asserted by the presence of a vaginal plug were used as foster mothers for the ES cell containing blastocysts. Development of the blastocysts continues in vivo and pups were generally born 16-18 days later. The contribution of the ES cells to the offspring could be judged visually by examination of the coat color of the pups. The blastocysts were obtained from C57BL/6 mice, which are solid black in color. The ES cell line E14TG2a, the parental line from which ES22a was derived was isolated from 129/Ola mice. This mouse strain is cream in color, the combined effect of three coat color genes, the dominant A^{w} allele at the agouti locus, recessive pink-eyed-dilute allele at the p locus and the recessive c^{ch} at the C locus. Offspring in which the ES22a had participated in the formation of the animal had coats containing brown and cream

hairs. About 80% of the pups from blastocysts containing ES22a cells showed some degree of coat color chimerism.

Generation of Animals Heterozygous for the Mutated β_2m Gene.

If ES22a cells contribute to the gonads the animals would be expected to generate sperm which contain the ES22a genome and pass it on to its offspring. The ES22a genome is homozygous for the dominant color coat marker A^W. If the chimera is mated with an animal that is non-agouti such as a C57BL/6 or B6/D2, offspring that arise from sperm or ES cell origin can be distinguished from those derived from sperm or blastocyst origin by their coat color. 50% of these agouti animals would be expected to inherit the mutated β_2m gene. These can be identified by analysis of DNA isolated from the tails. 1 cm of tail was therefore removed from the agouti animals, and DNA prepared by standard techniques. DNA was digested with either the restriction enzyme XbaI or HindIII and analyzed by Southern blotting and probing with a radioactively labelled fragment of the β_2m gene. The presence of an XbaI or HindIII fragment 1Kb larger than that found in control mice is indicative of the presence of the mutated β_2m gene in the animal.

Generation of Animals Homozygous for the Mutated β_2m Gene.

Male and female animals whose DNA indicated that they were carrying one copy of the mutated β_2m gene were mated. Offspring of these matings were again analyzed for the presence of the larger XbaI or HindIII fragments. As expected one quarter of the offspring from such matings were homozygous for the defective g ne. These animals now represent a new mouse strain which carries the mutation that was

originally introduced by homologous recombination into the ES cell E14TG2a.

Determination of the Phenotype of the β_2m -/- Mice

To determine whether as expected, the mutation of the β_2m protein resulted in loss of class I expression, two animals homozygous for the β_2m mutation were sacrificed and examined for the presence of cell surface class I expression. Cells isolated from lymph node, spleen and thymus were examined with monoclonal antibodies directed against the Class I antigens H-2K^b and H-2D^b. Both 129/Ola, the mouse strain from which the ES cell line was derived and C57BL/6 the strain with which the chimera giving rise to these animals had been mated, express the H-2^b haplotype. No staining above background was seen with cells obtained from the homozygous β_2m -/- mice in any of the tissues examined. Therefore, as predicted, the inactivation of β_2m gene resulted in an animal that fails to express Class I antigens at the cell surface. The animals appeared healthy and could not be distinguished visibly from their litter mates.

The effect of lack of class I antigens on the maturation of T-cells was examined by isolating and staining thymocytes with antibodies that delineate various stages of T-cell differentiation. The data showed that the CD4-8-, CD4+8+, and CD4+8- cell populations in the thymuses of normal, β_2m -/-, and heterozygous animals are identical. In contrast, the CD4-8+ populations differ between animals of the different genotypes. CD4-8+ cells represent 10% of the cells of the normal thymus but less than 1% of the cells in the thymus of the β_2m mice. Interestingly, the number of these cells in the heterozygote is also somewhat reduced.

To determine whether the absence of the Class I genes affected the maturation of T-cells as indicated by the expression of the T cell receptor

genes, thymocytes were stained with antibodies directed against either TCR$\alpha\beta$ or TCR$\gamma\delta$ receptor. No significant difference in the profile of $\alpha\beta$ cell receptor positive cells was seen in β_2m -/- animals compared to normal, indicating that Class I antigens are not needed for the maturation of thymocytes to TCR bearing CD4+8+, or CD4+8- cells.

Next, peripheral T-cells were examined for expression of $\alpha\beta$ TCR and CD4 and CD8. The yields of T-cells bearing $\alpha\beta$ TCRs from the spleen and lymph nodes of animals lacking β_2m were not significantly different from those of normal littermate controls. Between 20% and 32% of all T-cells bearing $\alpha\beta$ TCRs also bore CD8 in β_2m +/+ and +/- animals. Although CD4-, CD8+ thymocytes were somewhat depleted in β_2m heterozygous animals, the level of peripheral CD8+ T-cells in these mice were comparable to those of normal littermates. By contrast, virtually none of the $\alpha\beta$ TCR-bearing T-cells expressed CD8 in animals homozygous for the β_2m mutation. A preliminary experiment was done to find out whether the few $\alpha\beta$ T-cells which appeared CD8+ in mutant mice were due to noise in the staining procedures. T-cells from these animals were therefore grown for several days on plastic coated with anti-CD3 antibody and in interleukin-2, a procedure which often stimulates the proliferation of CD8+ T-cells preferentially. CD8 bearing $\alpha\beta$+ T-cells did not appear in greater numbers after such treatment, although $\gamma\delta$ bearing T cells did grow out. The conclusion is that CD8+, $\alpha\beta$ cells are virtually absent in animals which lack Class I MHC expression.

Thymocytes and T-cells from spleen and lymph node were also examined for expression of $\gamma\delta$ TCRs. The numbers of these cells were similar in β_2m -/- mice and controls. An outgrowth exp riment (described above) showed that the $\gamma\delta$-bearing cells from β_2m could proliferate and, moreover, preliminary

examination of these cells indicated that about a quarter of them bore CD8. Therefore these studies indicate that $\gamma\delta$ T-cells may not require Class I expression for their existence, even if they also bear CD8.

In accordance with the above results, cells can be provided which will not be subject to immune destruction as a result of the presence of Class I MHC antigens. The cells may find wide use, since they will not be subject to immune attack when introduced into an allogeneic host, while they will still be capable of functioning in their native manner. In this way, a wide range of diseases resulting from the loss of number and or function of cells may be treated, where the introduced cells will survive, multiply and function. Therefore, not only may diseases as a result of burns, abrasions, pathogens or the like be treated, but also diseases as a result of genetic defects.

Also, embryonic stem cells may be modified by homologous recombination to provide for chimeric mammalian hosts. The chimeric mammalian hosts may then be selected and used for breeding to produce homozygous hosts lacking the inactivated gene.

All publications and patent applications cited In this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually Indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scop of the appended claims.

WHAT IS CLAIMED IS:

1. A mammalian cell lacking at least one major histocompatibility complex (MHC) antigen as a result of introduction of a lesion at each of the loci of one subunit of said antigen by transformation of said mammalian cell with at least one DNA construct comprising a sequence homologous with at least a portion of said loci and said lesion and integration of said construct at said loci.

2. A mammalian cell according to Claim 1, wherein said MHC antigen is a Class I antigen.

3. A mammalian cell according to Claim 1, wherein said MHC antigen is a Class II antigen.

4. A mammalian cell lacking Class I major histocompatibility complex (MHC) antigens as a result of introduction of a lesion at each of the β_2-microglobulin loci by transformation of said mammalian cell with at least one DNA construct comprising a sequence homologous with at least a portion of said loci and said lesion and integration of said construct at said loci.

5. A mammalian cell according to Claim 4, wherein said cell is an epidermal cell.

6. A mammalian cell according to Claim 4, wherein said lesion is an insertion of a marker gene in the coding region of said β_2-microglobulin loci.

7. A mammalian cell according to Claim 6, wherein said marker gene is a n omycin resistance gene.

8. A mammalian cell according to Claim 4, wherein each of said loci have a different lesion.

9. A mammalian cell according to Claim 4, wherein said cell is murine.

10. A mammalian cell according to Claim 4, wherein said cell is human.

11. A method for producing a mammalian cell lacking at least one major histocompatibility antigen as a result of introduction of a lesion at each of the loci of a subunit of said antigen by transformation of said mammalian cell with at least one DNA construct comprising a sequence homologous with at least a portion of said locus and said lesion and integration of said construct at said loci, said method comprising:

transforming a host cell with said DNA construct, wherein said construct comprises a marker gene for selection of host cells comprising said DNA;

screening selected cells comprising said construct for homologous integration;

transforming cells having homologous integration with said construct; and

selecting cells lacking said MHC antigen on the surface by the absence of said MHC antigen.

12. A method according to Claim 11, wherein said MHC antigen is a Class I antigen.

13. A method according to Claim 12, wherein said locus is the β_2-microglobulin locus.

14. A method according to Claim 11, wherein said MHC antigen is a Class II antig n.

15. A method according to Claim 11, wherein said lesion is an insertion of a marker gene in at least one of said constructs.

16. A method according to Claim 13, wherein said selecting of cells lacking β_2-microglobulin is by using a cytotoxic agent specific for an epitope of a Class I MHC antigen.

17. A method according to Claim 11, wherein two different constructs are employed for the two stages of transforming.

18. A method according to Claim 17, wherein each of said constructs comprises a different marker for selection of transformed cells.

19. A method according to Claim 18, wherein one of said markers is resistance to neomycin and the other of said markers is sensitivity to acyclovir or gancyclovir.

20. Tissue comprising cells according to Claim 1.

21. Tissue according to Claim 20, wherein said cells are keratinocytes.

22. A method for treating a wound which comprises: administering, to said wound, tissue according to Claim 21.

23. A DNA construct comprising at least 50 bp of a sequence homologous with a locus of a subunit of an MHC antigen flanking a sequence encoding a marker gene capable of expression in a mammalian host.

24. A DNA construct according to Claim 23, wherein said MHC antigen is a Class I antigen.

25. A DNA construct according to Claim 23, wherein said MHC antigen is a Class II antigen.

26. A DNA construct comprising at least 50 bp of a sequence homologous with the β_2-microglobulin locus flanking a sequence encoding a marker gene capable of expression in a mammalian host.

27. A DNA construct according to Claim 26, wherein said marker gene is antibiotic resistance.

28. A DNA construct according to Claim 27, wherein said homology is at least in part with the coding region.

29. A DNA construct according to Claim 28, wherein said homology is at least 200 bp and comprises a region comprising exons 2 to 4 of said β_2-microglobulin locus.

30. An embryonic stem cell comprising a marker gene inserted into a chromosomal gene.

31. An embryonic stem cell according to Claim 30, wherein said marker gene is G418 resistance.

32. An embryonic stem cell wherein said marker gene is inserted into the β_2-microglobulin gene.

33. A method of producing chimeric non-human mammals, said method comprising:

transforming an embryonic stem c ll with a DNA construct comprising a marker gene and at

least 50 bp of DNA sequence homologous with a sequence of a target gene present in a chromosome of said embryonic stem cell under conditions where said construct becomes integrated by homologous recombination;

selecting for embryonic stem cells comprising said construct integrated into said target gene to provide selected cells;

introducing said selected cells into the blastocoel of a blastocyst of said mammal; and

growing said blastocyst into said chimeric mammal.

34. A method according to Claim 33, wherein said selecting is by means of said marker gene and polymerase chain reaction.

35. A method according to Claim 34, wherein said polymerase chain reaction employs two primers, one primer within said construct and one primer external to said construct but at the locus of said target gene.

36. A method according to Claim 33, wherein said target gene is β_2-microglobulin.

37. A non-human animal characterized by lacking Class I major histocompatibility complex antigens.

38. A animal according to claim 37, wherein said animal is a mammal.

39. A animal according to claim 38, wherein said mammal is a mouse.

40. A mouse characterized by the incapable of producing functional β_2-microglobulin, lacking Class I major histocompatibility complex antigens, and lacking mature CD8+ T lymphocytes.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/04178

I. CLASSIFICATI N OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 37/00; C12Q 1/68; C12N 5/00; C07H 15/12; A 01 H 5/00; C12N 15/00

U.S. Cl: 435/6; 435/240.2; 435/240.21; 536/27; 800/2; 424/93

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/6; 424/93; 536/27; 435/240.2, 240.21; 800/2

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

APA, CAS, BIOSYS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	Proceeding National Academy Sciences, Volume 84, issued April 1987, Korman et al. "Expression of human class II major histocompatibility complex antigens using retrovirus vectors", pages 2150-2154, see abstract.	23,25
X Y	Proceeding National Academy Sciences, Volume 85, issued October 1988, Chamberlain et al. "Tissue-specific and cell surface expression of human major histocompatibility complex class I heavy (HLA-B7) and light (B_2-microglobulin) chain genes in transgenic mice", pages 7690-7694, see abstract.	23,24,26,29 25,27,28
X Y	Proceedings National Academy Sciences, Volume 81, issued December 1984, Barbosa et al. "Recognition of HLA-A2 and -B7 antigens by cloned cytotoxic T lymphocytes after gene transfer human and monkey, but mouse, cells" pages 7549-7553, see abstract, page 7550.	23,24 25-29

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]

07 November 1990

Date of Mailing of this International Search Report [2]

03 JAN 1991

International Searching Authority [1]

ISA/US

Signature of Authorized Officer [20]

Scott A. Chambers

Form PCT/ISA/210 (second sheet) (May 1986)

III. DOCUMENTS C NSIDERED T BE RELEVANT (CONTINUED FROM THE SEC ND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
X Y	Journal Experimental Medicine, Volume 167, issued February 1988, Seong et al. "Rescue of Daudi Cell HLA Expression By Transfection of the Mouse B_2-microglobulin gene "pages 288-299, see abstract, page 289.	23,26-29 24,25
X Y	Journal of Immunology, Volume 141, issued 15 August 1988, Perarnau et al, "Human B_2-Microglobulin specifically enhance cell-surface expression of HLA class I molecules in Transfected Murine Cells," pages 1383-1389, see abstract, page 1384.	23,24,26-29 25
X	Nature, volume 338, issued 09 March 1989, Zimmer et al. "Production of chimaeric mice containing embryonic stem (ES) cells carrying a homoeobox Hox 1.1 allele mutated by homologous recombination, "pages 150-156, see abstract.	30,31,33-35
X Y	Nature, volume 336, issued 24 November 1988, Mansaur et al. "Disruption of the proto-oncogene int-2 in mouse embryoderived stem cells: a general strategy for targeting mutations to non-selectable genes" pages 348-352, see entire document.	30,31 33-35

FURTHER INFORMATION CONTINUED FROM THE SEC ND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See attachment to Telephone Memorandum

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable of the international application. TELEPHONE PRACTICE

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment To PCT/IPEA/210
PCT TELEPHONE MEMORANDUM

I Claims 1-22 drawn to mammalian cells lacking MHC antigens, a method for making said cells and a method for using tissue comprising said cells to treat wounds, classified in Class 435, subclass 240.2 and Class 424, subclass 95;

II Claims 23-29 and 33-36 drawn to a DNA construct and a method for making chimeric mammals using a similar DNA construct, classified in Class 536, subclass 27;

III Claims 30-32 drawn to transformed embryonic cells classified in Class 435, subclass 252.3;

IV. Claims 37-40 drawn to nonhuman mammals lacking Class I MHC antigens, classified in Class 435, subclass 252.3.

The claims of these four groups are drawn to inventions which are related but separate and not linked in the claims.